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Absence of Detectable Measles Virus Genome Sequence in Inflammatory Bowel Disease Tissues and Peripheral Blood Lymphocytes

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A highly sensitive measles-specific RT-PCR-nested PCR system was established, which consistently amplified measles virus genome sequence from control samples containing as little as 5.5×10^{-3} pfu per reaction. This method failed to detect the presence of measles virus in 93 colonoscopic biopsies and 31 peripheral blood lymphocyte preparations, examined and obtained from patients with inflammatory bowel disease (IBD) and noninflammatory controls. All patients had detectable levels of serum neutralization antibody against measles virus. Each biopsy was estimated to have about one million cells, based on the amplification of the beta actin gene. The assay was calibrated by use of a known number of lymphocytes. The method applied was able to amplify measles virus RNA from a nucleic acid mixture equivalent to 18 cells derived from subacute sclerosing panencephalitis (SSPE) brain material. The level of measles RNA present, if any, in the biopsies is therefore at least 50,000-fold less than in SSPE. ***J. Med. Virol. 55:243–249, 1998.***

KEY WORDS: measles; RT-PCR; Crohn's disease

INTRODUCTION

The etiology of inflammatory bowel disease (IBD) is not yet known. In the past, several pathogens, including *Yersinia, Brucella, Mycobacteria, Chlamydias, Cytomegalovirus, Herpesvirus, E. coli, Streptococcus,* and *Listeria,* have been considered putative agents [Blaser et al., 1984a, 1984b; Wakefield et al., 1992; Wayne et al., 1992; Cartan et al., 1993; Liu et al., 1995] but none has been definitively characterized as the causative organism. Genetic predisposition to the development of Crohn's disease through paternal transmission has also been suggested [Polito et al., 1996]. Recently, it has been reported that IBD (Crohn's disease and ulcerative colitis) is linked to the presence of measles virus and previous measles vaccination. Originally, it was hypothesized that perinatal exposure to wild-type measles virus may lead to the establishment of Crohn's disease later in life [Ekbom et al., 1994, 1996]. Similarly, immunization with live attenuated measles virus vaccine was also proposed as a risk factor [Thompson et al., 1995]. These findings were mostly drawn from studies relating to epidemiology and case control observations of the disease. However, the presence of measles virus nucleic acid and nucleocapsid protein components was reported in the gut tissues of IBD patients [Wakefield et al., 1993, 1997]. The interpretation of the results is controversial and has been the subject of critical scientific comments [Iizuka et al., 1995; Haga et al., 1996; Feeney et al., 1997; Fisher et al., 1997; Metcalf 1998; Nielsen et al., 1998].

Measles induces immune suppression and the mechanism by which measles virus can establish persistent infection is not known. Generally, the acute disease is self-limiting and provides life-long immunity. The virus tropism in acute measles is directed largely through the epithelial cells of the respiratory tract and cells of the immune system, i.e., lymphocytes and macrophages [Casali et al., 1989]. Previously, measles virus has been shown to be present in the mucosal gut and surrounding tissues, such as the lymph nodes and appendix [Fournier et al., 1986]. While in theory measles virus could establish persistent infection in these organs, the continued presence of measles-specific nucleic acid has not been demonstrated.

In this study we attempted to amplify the measles virus genome sequence from clinical specimens ob-

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tained from patients with IBD and noninflammatory controls with a view to detecting the presence of measles-virus-specific nucleic acid and also to characterize measles virus strains, if any, present in the gut tissues and peripheral blood lymphocytes. By using an exquisitely sensitive, measles-specific RT-PCR-nested PCR system we examined 93 colonoscopic biopsies and 31 preparations of lymphocytes. None of the clinical specimens examined produced measles-specific DNA fragments. The system established successfully amplified measles-specific RNA from nucleic acid extracted from brain tissue persistently infected with measles virus in the form of SSPE.

PATIENTS AND METHODS

Thirty patients with inflammatory bowel disease or non-IBD controls were recruited prospectively from routine colonoscopy lists. In each case the diagnosis had been confirmed by standard clinical radiological and histological criteria. Measles vaccination and illness histories were recorded and these were subsequently confirmed by the general practitioners.

At colonoscopy, three biopsies were taken at sites >20 cm apart; in patients with active disease, specimens were obtained from both macroscopically normal and inflamed mucosa. If an apthoid ulcer was present, a biopsy was taken from the ulcer edge. A stringent specimen collection protocol was followed to avoid contamination and new disposable biopsy forceps were used for each patient. All biopsies were snap frozen in liquid nitrogen and stored at −70°C. Verbal consent was obtained from all patients and the study was approved by Lothian research ethics committee.

SSPE Brain Material

Partially homogenized brain material (96/33839) was received from Dr. Lee Jin, PHLS, Colindale, London. This material was originally collected in Belfast, Ireland, from a patient who died from subacute sclerosing panencephalitis (SSPE).

Measles Virus Strains

Wild-type measles virus strain (94/31825) was also received from PHLS, Colindale, London. This strain has previously been designated as UK/64, it belongs to genotype group II [Jin et al., 1996]. The strain was propagated at NIBSC in Vero cells and the stock has a titer of 5.5×10^4 pfu/ml. Measles strain Philadelphia 26 (PH26) was used as a challenge virus during the serum neutralization antibody assay. The assay was carried out essentially as described for mumps virus previously [Afzal et al., 1997].

Lymphocyte Preparation

Nine ml of citrated blood was added to 7 ml of RPMI-1640 (Sigma, St. Louis, MO) medium. This was layered onto Ficoll (Histopaque-1077 Sigma) and centrifuged at 1,600 rpm for 30 minutes. The buffy layer was removed and added to 10 ml of RPMI-1640. This was centrifuged at 1,600 rpm for 15 minutes and the resultant pellet was resuspended in 1 ml of Tris-EDTA and stored at −70°C. Total cells were estimated by counting a fraction (1 μl) from six randomly selected preparations, which showed that, on average, each ml of the final preparation had approximately 4.2×10^6 cells.

Oligonucleotide Primers

Measles virus N gene primers (MV1, MV2, MV3, MV4) and the human beta actin gene primers (AC1, AC2) have been described elsewhere [Ray et al., 1996]. Measles virus M gene primers had the following sequences: MV13 5′-GCGACAGGAAGGATGAATGC-3′ (mRNA sense, positions 3565–3584); MV14 5′-GTTTGCGTTGAAGACACTCC-3′ (Genome sense, positions 3832– 3851); MV15 5′-TATGTACATGTTTCTGC-3′ (mRNA sense, positions 3587–3603); and MV16 5′-GTTGTTAGGACCTTTCTCC-3′ (Genome sense, positions 3811–3829). All positions correspond to the measles virus sequence reported under accession number k01711 in the EMBL sequence database.

RNA Extraction

Total cellular RNA was extracted from clinical biopsies, peripheral blood lymphocyte preparations, and positive control samples by using the RNAgents kit (Promega). Briefly, the clinical material was transferred into a screw-top tube containing 10–12 glass balls, size 2.5 to 3.5 mm (BDH) and 350 μl of denaturing solution (part of the kit). The tube was then allowed to shake in a Mini-Beadbeater (Biospec Products) at 4,600 rpm for 5 seconds at room temperature. This process was repeated three times or more, until the entire tissue was completely dissolved. After addition of 35 μl of 2-M sodium acetate pH 4.0 and 350 μl of phenol-chloroform-isoamyl alcohol mixture, the nucleic acid was extracted according to the instructions provided with the kit. Finally, the nucleic acid pellet was dissolved in 14-μl nuclease-free water, and 13 μl was used for measles-specific RT-PCR while the remaining 1 μl was used for the amplification of human beta actin gene-specific DNA fragments by PCR.

RT-PCR Methods

In order to select the most sensitive measles-specific reverse transcriptase-polymerase chain reaction (RT-PCR) method, initially, two approaches were applied. In the first approach (two-step approach) cDNA synthesis of the control RNA templates was performed as an independent step using random primers (dN6) or the measles N gene primers (MV1 or MV2) and AMV-reverse transcriptase enzyme (NBL Gene Sciences). In the second step, cDNA copies were amplified by PCR in the presence of MV1/MV2 primers and AmpliTaq DNA polymerase enzyme for 30 cycles (94°C for 1 min; 50°C for 0.5 min; 72°C for 1 min).

In the second approach (single-step approach), both reverse transcription and primary PCR steps were combined by using the EZ rTth RNA PCR kit (Perkin Elmer, Norwalk, CT). This system does not allow the use of random primers. Routinely, RNA templates,

TABLE I. Clinical Details of Patients With IBD and Controls

Patient group	Number of patients	Age at procedure mean, years (range)	Age at diagnosis mean, years (range)	Sex, M:F	Measles vaccination (GP confirmed)	Steriods	Average antibody titer
Crohn's disease	9	37 (20–64)	31 (18–42)	3:6	4/9 (2/9)	3/9	1:150
Ulcerative colitis	9	42 (27–62)	38 (21–56)	3:6	4/9 (2/9)	4/9	1:245
Indeterminate Colitis	1	59	51	0:1	0	0	1:252
Non-IBD controls	11	48 (30–69)		7:4	2/11 (1/11)	0	1:444

both primers (MV1/MV2), EZ buffer, rTth DNA polymerase (5 units/reaction), and other essential reagents were added together and reaction tubes were processed as follows. cDNA synthesis was carried out at 60°C for 30 minutes on a thermal cycler (Cyclogene, Techne). This step was linked to the following: (1) heating to 94°C for 2 minutes, (2) amplification of cDNA for 40 cycles (94°C for 45 sec, 60°C for 45 sec per cycle), and (3) final template elongation at 60°C for 7 min. All steps were performed according to the instructions supplied with EZ rTth RNA PCR kit (Perkin Elmer).

Nested PCR

All primary PCR products, whether generated by conventional RT-PCR or by rTth RT-PCR methods, were subjected to nested PCR amplifications using 2 μl of primary RT-PCR products, MV3 and MV4 primers, dNTP mixture containing digoxigenin-11-dUTP (Boehringer Mannheim, Germany), and AmpliTaq DNA polymerase (0.5 units/reaction). All nested PCR amplifications involved 30 cycles (94°C for 1 min; 50°C for 0.5 min; 72°C for 1 min).

Agarose Gel Analysis, Digoxigenin Antibody Assay, and Southern Blotting

A volume of (8/100 μl) nested PCR product was resolved in 1% agarose gel containing ethidium bromide and DNA products were visualized by UV transilluminator. Agarose gels were photographed and the products transferred to a nylon membrane (Hybond-N, Amersham, UK) by capillary blotting in 10 × SSC. Gels used for Southern blots were denatured and neutralized prior to blotting [Sambrook et al., 1989], while those used for digoxigenin antibody assay were blotted without any treatments. Membranes were challenged with measles virus N gene-specific oligonucleotide probe (MV3) radiolabeled with gamma-p^{32}-ATP (ICN) by DNA polynucleotide kinase enzyme. Digoxigenin antibody assay was carried out using the Dig. nucleic acid detection kit (Boehringer Mannheim).

Amplification of Human Beta Actin Gene DNA

A portion of the nucleic acid mixture extracted from clinical samples was used for the amplification of the human beta actin gene-specific DNA fragments with AmpliTaq Gold DNA polymerase in the presence of AC1 and AC2 primers and other standards reagents. Prior to PCR, the reaction mixtures were incubated at 94°C for 10 minutes to inactivate the antibody bound to AmpliTaq Gold DNA polymerase. The amplification was then carried out for 40 cycles (94°C for 1 min; 50°C for 0.5 min; 72°C for 1 min). The amounts of DNA products generated from each sample were quantified by dot blot analysis by using the actin gene-specific oligonucleotide probe, AC1, radiolabeled with gamma-p^{32}-ATP (ICN). The blot was monitored and counted by Instantimager (Packard).

RESULTS

From each patient three colonoscopic biopsies and one lymphocyte preparation were examined for the presence of measles virus genome sequence by rTth RT-PCR-nested PCR method. Specimens from one patient were taken twice with an interval of three months. Therefore, a total of 93 colonoscopic biopies and 31 lymphocyte preparations were examined during this study. In addition, 31 blood serum samples were also tested to identify the presence of measles-specific neutralizing antibodies in the blood. All patients had detectable levels of serum neutralization antibodies against measles virus (Table I). The average antibody titer of all groups was 1:197. Only 5 patients had a confirmed history of measles vaccination.

Sensitivity of RT-PCR-Nested PCR Assays

Conventional RT-PCR involved the use of random primers to generate cDNA with reverse transcriptase, followed by PCR with specific primers in a second reaction. rTth RT-PCR required a single tube in which cDNA was synthesized by the same enzyme mix that was used in the subsequent PCR steps. rTth RT-PCR used the same specific primers to generate cDNA as were used in the PCR. For both conventional RT-PCR and rTth RT-PCR, the products were further amplified by a nested PCR to increase sensitivity. When a dilution series of stock measles virus was assayed by conventional RT-PCR, nucleic acid was detected from the equivalent of 5.5 pfu while rTth RT-PCR detected nucleic acid from 0.55 pfu as shown in Table II. As shown there and also reported by Chadwick et al. [1998], rTh RT-PCR is therefore more sensitive than the conventional method.

Extraction of nucleic acid from tissues may either give reduced sensitivity if the template is degraded or the tissue contains inhibitors, or an increased sensitivity if the nucleic acid from the cells acts as carrier molecule to improve extraction and precipitation efficiency. Serial 10-fold dilutions of the measles stock vi-

TABLE II. Findings of RT-PCR-Nested PCR Amplifications of Control RNA Templates*

S. no	Virus dilution (pfu)/reaction	A: conventional RT-PCR + nPCR	B: rTth PCR + nPCR	C: conventional RT-PCR + nPCR	D: rTth PCR + nPCR
1	55	+	+	+	+
2	5.5	+	+	+	+
3	5.5×10^{-1}	–	+	+	+
4	5.5×10^{-2}	–	–	–	+
5	5.5×10^{-3}	–	–	–	+
6	5.5×10^{-4}	–	–	–	–
7	water	–	–	–	–

*A and B: target RNA templates extracted in the absence of carrier lymphocytes. C and D: target RNA templates extracted in the presence of carrier lymphocytes.

rus were extracted in the presence of 20-μl lymphocytes. Conventional RT-PCR-nested PCR gave an end point of 0.55 pfu, while rTth RT-PCR gave an end point of 5.5×10^{-3} pfu (Table II, Fig. 1B). The addition of tissue therefore increased the sensitivity. Similar end points were observed with the digoxigenin antibody and Southern blot assays, although detection of DNA products in control experiments by either of these methods is 10-fold more sensitive than ethidium bromide staining. In subsequent experiments, some clinical specimens, randomly selected, were spiked with measles virus prior to RNA extraction. All spiked samples gave bands of a comparable intensity, suggesting that none of the tissues either contained inhibitors or significantly degraded the RNA (Fig. 1A).

Measles Sequences Detection in SSPE Brain Material

Serial 10-fold dilutions of total cellular nucleic acid extracted from SSPE brain material were amplified using various protocols, and the results are shown in Figure 2. In all cases, detection was by ethidium bromide staining. Based on total cell counts using a hemocytometer, dilution 1 corresponded to 1.8×10^4 cells. Conventional RT-PCR-nested PCR using random primers gave a band only in dilution 1, while the use of either MV1 or MV2, the measles-specific primers, in the RT step gave a band down to dilution 2. However, rTth RT-PCR-nested PCR gave bands to dilution 4, corresponding to 18 cells. As with the assay of free virus in the presence of added cells, rTth RT-PCR was 1,000-fold more sensitive than RT-PCR with random primers. This gives some confidence in the applicability of the assay to infected rather than spiked tissue and demonstrates its high sensitivity. Primers for the M gene were of the same sensitivity as for the N gene by rTth RT-PCR-nested PCR (data not shown).

Quantification of Cell Numbers in Biopsies by Assay of Human Beta Actin Gene DNA

The colonoscopy specimens were all of similar size, but the number of cells could not be estimated by hemocytometer as they were pieces of solid tissue and were extracted directly with the denaturing solution. Cells were therefore quantified by amplifying the beta actin gene DNA and comparing the signals obtained with those from known numbers of lymphocytes.

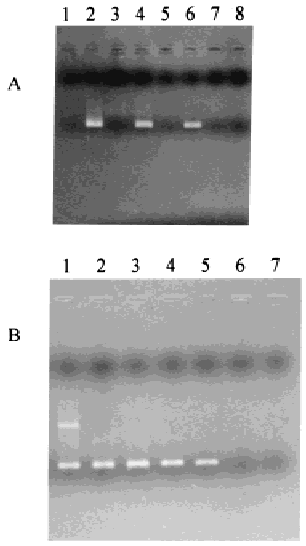

Fig. 1. **A:** Nested RT-PCR amplification products of RNA templates originally extracted from (1) 100-μl lymphocytes; (2) 100-μl lymphocytes + 55 pfu of measles stock virus; (3) half of a biopsy sample; (4) half of a biopsy sample + 55 pfu of measles stock virus; (5) 100-μl distilled water; (6) 100-μl distilled water + 55-pfu measles stock virus; and (7) and (8) distilled water only. **B:** rTth RT-PCR-nested PCR amplification products of RNA templates originally extracted from 20-μl lymphocytes, which were spiked with (1–6) 10-fold serial dilution of measles stock virus. The first dilution had 55 infectious virus particles; sample 7 had 20-μl lymphocytes only.

Nucleic acid was extracted from lymphocytes and from biopsy specimens, and serial 10-fold dilutions were made and amplified by PCR. The results are presented in Figure 3A for the lymphocytes, where dilution 1 represents 10^4 cells, and in Fig. 3B, where dilution 1 represents one-tenth of the total biopsy. The last positive dilutions were dilution 4 for lymphocytes corresponding to 10 cells and dilution 5 for the biopsy. Thus the number of cells in the total biopsy is approximately one million. The quantity of product detected did not titrate with the dilution in a linear manner, although end points of detection could be determined.

RT-PCR-Nested PCR of Clinical Specimens

RNA templates extracted from colonoscopic biopsies and lymphocyte preparations were amplified by the rTth RT-PCR method. All primary PCR products were reamplified by nested PCR in the presence of digoxigenin-labeled dNTPs mixture, MV3/MV4 primers, and

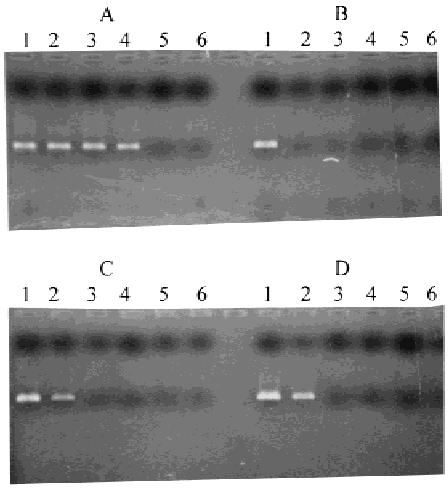

Fig. 2. Nested RT-PCR amplification products of a nucleic acid mixture extracted from SSPE brain material. Serial 10-fold dilutions (samples 1–5) of nucleic acid mixture were amplified by: rTh RT-PCR-nested PCR **(A);** cDNA synthesis with random primers followed by conventional RT-PCR-nested PCR **(B);** cDNA synthesis with MV1 primers followed by conventional RT-PCR-nested PCR **(C);** and cDNA synthesis with MV2 primers followed by conventional RT-PCR-nested PCR **(D).** Sample 6 had distilled water only.

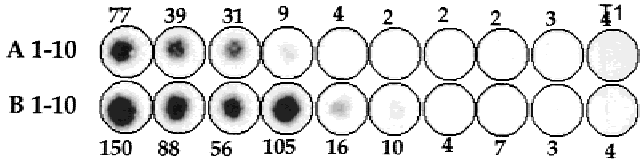

Fig. 3. Dot blot assay of the DNA products amplified from the human beta actin gene-specific DNA templates. **A:** Serial 10-fold dilutions (A1–10) of nucleic acid mixture, A1 corresponds to 1×10^4 lymphocytes. **B:** Serial 10-fold dilutions (B1–10) of a fraction of nucleic acid (1/10) extracted from a colonoscopic biopsy. Radioactive counts are shown at the top or bottom of each circle.

AmpliTaq DNA polymerase. All nested PCR products were examined by agarose gel analysis, digoxigenin antibody assay, and Southern hybridization. An example of the assay is shown in Figure 4.

None of the RT-PCR-nested PCR amplifications of the nucleic acids extracted from clinical biopsies and peripheral blood lymphocytes produced DNA fragments of the size expected for measles-specific sequences (252 bp). All experiments had appropriate positive and negative controls in each assay run, including detection of beta actin sequences.

False Positive and Cross-Contamination

Nonspecific DNA bands were identified in RT-PCR-nested PCR amplification products of the clinical samples. They were only visible in the digoxigenin antibody assay (marked by an arrow in Fig. 4B). In Southern blots these bands did not react with the measles N gene-specific probe (Fig. 4C). Concentrated products of the bands were produced by precipitating the entire nested PCR products with isopropanol. These products could not be sequenced by using the measles N gene-specific primers (data not shown).

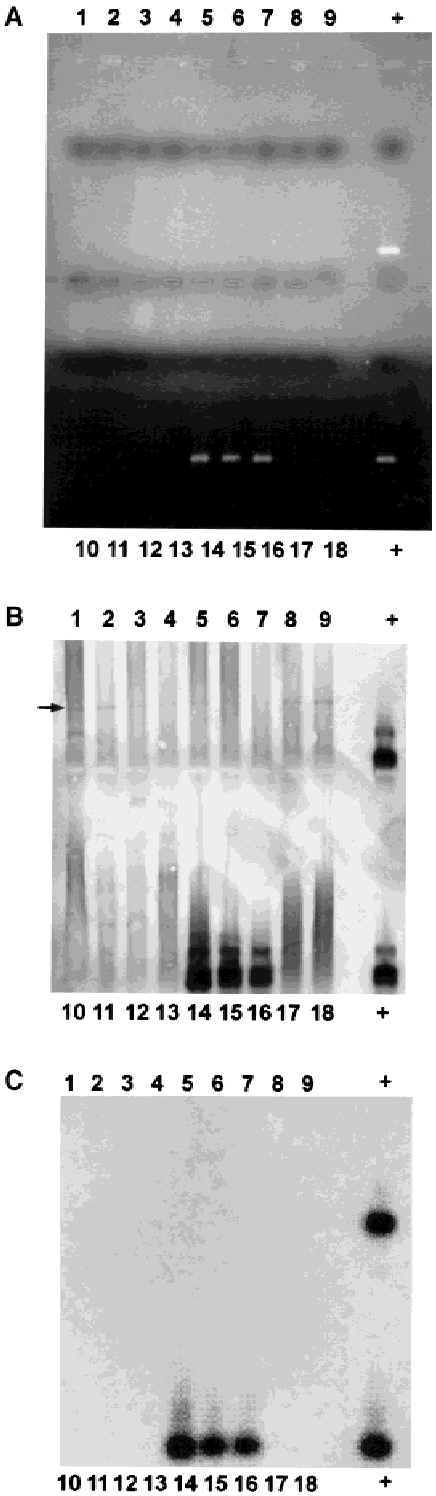

Fig. 4. rTth RT-PCR-nested PCR amplification products of RNA templates originally derived from: (1–13) colonoscopic biopsies; (14) SSPE brain material; (15) 10-μl lymphocytes + 5.5×10^{-1} virus particles; (16) 10-μl lymphocytes + 5.5×10^{-2} virus particles; (17) 10-μl lymphocytes only; and (18) water, negative control. + denotes positive control, measles-specific DNA products used as a size marker. **A:** Agarose gel analysis by ethidium bromide staining; **B:** Digoxigenin antibody assay; **C:** Southern hybridization using measles-specific radiolabeled oligonucleotide probe.

One biopsy sample produced DNA fragments of the size expected for a measles sequence. However, four other specimens from this patient, including two colonoscopic biopsies, one lymphocyte preparation, and one serum sample, remained negative. The DNA product of the positive sample was sequenced and the 218 bases generated (corresponding to positions 1263–1481, accession number k01711) were compared with similar sequences generated from positive controls. Nucleotide sequence generated from the biopsy sample was identical to the sequence derived from the 94/31825 measles strain. This strain was routinely used as one of the positive controls during this study. The sequence derived from SSPE brain material differed by three nucleotides from 94/31825 sequence in this region of the genome. It was therefore concluded that the DNA product generated from the biopsy sample originated through cross-contamination with the positive control.

DISCUSSION

The 30 patients examined in this study had a wide range of clinical manifestations and age distribution. Serum antibody levels determined by the virus neutralization assay showed that all patients had either been exposed to measles wild type or vaccine strains previously. The antibody titers of the non-IBD control group were similar to the titer of the Crohn's group, while the antibody titer of ulcerative colitis group, although slightly higher, did not differ statistically from the average titer of this study. Previous serological studies have shown that SSPE cases, where measles persistence is well established, had significantly higher levels of measles-specific antibodies both in serum and CSF [Connolly et al., 1967; Jabbour and Sever, 1968]. Such an increase in the antibody levels was not observed in the serum samples collected from IBD patients. Our findings are in agreement with Fisher et al. [1997].

The data presented in this report clearly demonstrate that rTth RT-PCR-nested PCR is at least 100- to 1,000-fold more sensitive than reverse transcription followed by PCR and nested PCR. In our hands the method has been able to identify the presence of measles virus genome sequence in control samples that had measles virus corresponding to as little as 5.5×10^{-3} pfu. The sensitivity limit of the assay in terms of genome copy numbers can not be defined at this stage. Measles N gene primers used during this study are reported to be highly sensitive and would be expected to amplify the target sequence from all known strains of measles virus [Ray et al., 1996]. In addition, nonsegmented negative strand RNA viruses are known to generate a transcription gradient of mRNA species from the 3′–5′ ends of the genome. The N protein gene is located at the 3′ end, and, therefore, its mRNA species are transcribed much more efficiently [Cattaneo et al., 1987], a gradient that is often exaggerated in persistent infections [Sidhu et al., 1994]. These factors essentially make the N gene sequence a suitable target for the amplification of low copy number RNA species. In addition, the technology applied uses both primers during cDNA synthesis, suggesting that both plus and minus sense RNA templates should have been amplified from the clinical tissues.

In each assay run, DNA templates of the human beta actin gene DNA molecules were amplified by PCR from the nucleic acid mixture of biopsy samples and lymphocytes. The amount of resultant DNA product was calibrated against the DNA products generated from a known number of lymphocytes, which demonstrated that about one million cells were examined per biopsy. When samples from SSPE were examined, measles-specific nucleic acid could be detected from a sample equivalent to 18 cells. In contrast, no sample from IBD or control biopsies or lymphocytes gave a positive signal. The amount of measles-specific RNA, positive and negative sense, is therefore more than 50,000-fold lower in the colonoscopic specimens than in SSPE, consistent with the view that no measles-specific nucleic acid is present in the gut tissues. Others have similarly failed to detect measles sequences by PCR [Haga et al., 1996]. It seems unlikely that other methods, including transmission electron microscopy, immuno-gold staining, and in situ hybridization, which have shown the presence of measles virus in IBD tissues [Wakefield et al., 1993, 1997], could have examined sufficient cells to detect measles antigen expressed from such a low level of measles RNA, and it is possible that the positive findings reported are artifacts of immune staining either because of cross-reaction of the antibody or nonspecific adsorption [Iizuka and Masamune 1997].

While all colonoscopic biopsies and lymphocytes preparations examined by RT-PCR-nested PCR were negative, one biopsy sample was cross-contaminated with the positive control at the primary PCR level. In addition, DNA bands identified in the digoxigenin antibody assay did not show any reactivity with measles-specific probe and could not be sequenced with the measles primers. The origin of these bands in RT-PCR-nested PCR amplification products with measles N gene-specific primers cannot be explained at this stage.

Our experience also demonstrates that even with the most scrupulous attention to methodology and laboratory procedures, cross-contamination of specimens can occasionally occur, so that considerable experience and a range of methodologies may be needed to recognize nonspecific reactions that might be interpreted as positive if confirmatory techniques are not applied.

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REFERENCES

Afzal MA, Buchanan J, Dias JA, Cordeiro M, Bentley ML, Shorrock CA, Minor PD (1997): RT-PCR based diagnosis and molecular characterization of mumps viruses derived from clinical specimens collected during the 1996 mumps outbreak in Portugal. Journal of Medical Virology 52:349–353.

Blaser MJ, Hoverson D, Ely IG, Duncan DJ, Wang WL, Brown WR

(1984a): Studies of *Campylobacter jejuni* in patients with inflammatory bowel disease. Gastroenterologoy 86:33–38.

Blaser MJ, Miller RA, Lacher J, Singleton JW (1984b): Pateints with active Crohn's disease have elevated serum antibodies to antigens of seven enteric bacterial pathogens. Gastroenterologoy 87:888–894.

Casali P, Nakamura M, McChesney MB (1989): Immunosuppression by measles virus. In Spector S, Bendinelli M, Friedmann H (eds): "Virus-Induced Immunosuppression." New York: Plenum Press, pp 345–373.

Cartun RW, Van-Kruiningen HJ, Pedersen CA, Berman MM (1993): An immunocytochemical search for infectious agents in Crohn's disease. Modern Pathology 6:212–219.

Cattaneo R, Rebmann G, Schmid A, Baczko K, ter Meulen V, Billeter MA (1987): Altered transcription of a defective measles virus genome derived from a diseased human brain. EMBO Journal 6: 681–688.

Chadwick N, Bruce I, Davies M, van Gemen B, Schukkink R, Khan K, Pounder R, Wakefield A (1998): A sensitive and robust method of measles RNA detection. Journal of Virological Methods 70:59–70.

Connolly JH, Allen IV, Hurwitz LJ, Millar JH (1967): Measles virus antibody and antigen in subacute sclerosing panencephalitis. Lancet i:542–544.

Ekbom A, Wakefield AJ, Zack M, Adami HO (1994): Perinatal measles infection and subsequent Crohn's disease. Lancet 344:508–510.

Ekbom A, Daszak P, Kraaz W, Wakefield AJ (1996): Crohn's disease after in-utero measles virus exposure. Lancet 348:515–517.

Feeney M, Clegg A, Winwood P, Snook J (1997): A case-control study of measles vaccination and inflammatory bowel disease. Lancet 350:764–766.

Fisher NC, Yee L, Nightingale P, McEwan R, Gibson JA (1997): Measles virus serology in Crohn's disease. Gut 41:66–69.

Fournier J, Lebon P, Bouteille M, Goutieres F, Rozenblatt S (1986): Subacute sclerosing panencephalitis: Detection of measles virus RNA in appendix lymphoids tissue before clinical signs. British Medical Journal 293:523–524.

Haga Y, Funakoshi O, Kuroe K, Kanazawa K, Nakajima H, Saito H, Murata Y, Munakata A, Yoshida Y (1996): Absence of measles viral genomic sequence in intestinal tissues from Crohn's disease by nested polymerase chain reaction. Gut 38:211–215.

Iizuka M, Nakagomi O, Chiba M, Ueda S, Masamune O (1995): Absence of measles virus in Crohn's disease. Lancet 345:199.

Iizuka M, Masamune O (1997): Measles vaccination and inflammatory bowel disease. Lancet 350:1775.

Jabbour JT, Sever JL (1968): Serum measles antibody titers in patients with subacute sclerosing panencephalitis. Journal of Pediatrics 73:905–907.

Jin L, Richards A, Brown DW (1996): Development of a dual-target-PCR for detection and characterization of measles virus in clinical specimens. Molecular and Cellular Probes 10:191–200.

Liu Y, Van-Kruiningen J, West AB, Cartun RW, Cortot A, Colombel JF (1995): Immunocytochemical evidence of *Listeria, Escherichia coli,* and *Streptococcus* antigens in Crohn's disease. Gastroenterology 108:1396–1404.

Metcalf J (1998): Is measles infection associated with Crohn's disease? British Medical Journal 316:166.

Nielsen LLW, Nielsen NM, Melbye M, Sodermann M, Jacobsen M, Aaby P (1998): Exposure to measles virus in utero and Crohn's disease: Danish register study. British Medical Journal 316:196–197.

Polito JM 2nd, Rees RC, Childs B, Mendeloff AI, Harris ML, Bayless TM (1996): Preliminary evidence for genetic anticipation in Crohn's disease. Lancet 347:798–800.

Ray R, Cooper PJ, Sim R, Chadwick N, Earle P, Dhillon AP, Pounder RE, Wakefield AJ (1996): Direct in situ reverse transcriptase-polymerase chain reaction for detection of measles virus. Journal of Virological Methods 60:1–17.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning, A Laboratory Manual." Cold Spring, NY: Cold Spring Harbor Laboratory Press.

Sidhu MS, Crowley J, Lowenthal A, Karcher D, Menonna J, Cook S, Udem S, Dowling P (1994): Defective measles virus in human subacute sclerosing panencephalitis brain. Virology 202:631–641.

Thompson NP, Montgomery SM, Pounder RE, Wakefield AJ (1995): Is measles vaccination a risk factor for inflammatory bowel disease? Lancet 345:1071–1074.

Wakefield AJ, Fox JD, Sawyerr AM, Taylor JE, Sweenie CH, Smith M, Emery VC, Hudson M, Tedder RS, Pounder RE (1992): Detection of herpesvirus DNA in the large intestine of patients with ulcerative colitis and Crohn's disease using the nested polymerase chain reaction. Journal of Medical Virology 38:183–190.

Wakefield AJ, Pittilo RM, Sim R, Cosby SL, Stephenson JR, Dhillon AP, Pounders RE (1993): Evidence of persistent measles virus infection in Crohn's disease. Journal of Medical Virology 39:345–353.

Wakefield AJ, Sim R, Akbar AN, Pounders RE, Dhillon AP (1997): In situ immune responses in Crohn's disease: A comparison with acute and persistent measles virus infection. Journal of Medical Virology 51:90–100.

Wayne LG, Hollander D, Anderson B, Sramek HA, Vadheim CM, Rotter JI. (1992): Immunoglobulin A (IgA) and IgG serum antibodies to mycobacterial antigens in Crohn's disease patients and their relatives. Journal of Clinical Microbiology 30:2013–2018.